EFFECT OF SUBLETHAL DOSES OF AN AZO DYE, ACID BLUE-113 (AB -113) ON BIOCHEMICAL RESPONSES OF Labeo rohita AND Cirrhinus mrigala

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Received April 2, 2016 Accepted December 2, 2016

ABSTRACT
Present study envisaged to evaluate effect of an azo dye, Acid blue -113 (AB-113) (CI: 26360) on the survival and biochemical changes in the flesh of fry of two important food fishes Labeo rohita and Cirrhinus mrigala. This dye was observed to be very toxic as 96h LC50 came to be 0.54 mg/L for L. rohita and 1.73 mg/L for C. mrigala. Fry became restless and tried to jump out of water. Fins, gills and mouth of the fish absorbed dye in a dose dependent manner. Antioxidant/detoxification enzymes such as glutathione-s-transferase (GST), glutathione reductase (GR) and glutathione peroxidase (GPx) were determined in the flesh of L. rohita and C. mrigala after exposure to AB-113 dye. The highest sensitivity was shown by GR in both L. rohita and C. mrigala. The results indicate that the dye is very toxic to the fish and causes significant biochemical changes even at very low doses.

Key Words : Acid blue-113, Antioxidant enzymes, Azo dye, Labeo rohita, Cirrhinus mrigala

INTRODUCTION
Azo dyes are very toxic to the flora and fauna of the receiving water bodies and are characterized by one or more R-N=N-R (azo) bonds. These dyes comprise approximately 65% of the dyes used in textile, rubber products, enamel, plastic, printing ink, pharmaceutical, brewing, food, cosmetic, leather and paper industries. It is estimated that worldwide these industries discharge around 280,000 tons of dyes into the environment every year.1 Generally, a substantial amount of unbound dye is lost in waste water. For example, release of 50% of reactive dyes and 2% of basic dyes wastewater has been reported. All living beings are exposed to such potentially toxic azo dyes in the environment but their maximum impact is observed in aquatic organisms as almost all the pollutants ultimately reach aquatic bodies with the waste water or run off from industrial, domestic or agricultural sectors. These pollutants cause both acute and chronic damage to the DNA and proteins of aquatic organisms especially fish as they cannot escape the detrimental effects of such pollutants.2 At the same time even a very small amount of dye in water (10-50 mg/L) affects the transparency and gas solubility of water. The aquatic environment is therefore of primary concern for ecological risk assessment and environmental monitoring. Many toxicants induce a steady rise in the concentration of reactive oxygen species (ROS) and oxidative stress in aquatic organisms especially when the rate of ROS generation exceeds the rate of their degradation by cellular defense mechanisms. One approach that has been considered useful for evaluating effects of pollutants in water is to identify biomarkers of stress in fish. These biomarkers can provide an early warning of adverse effects before the onset of serious pathological damage. The antioxidative/detoxification processes play an important role in helping the fish to adapt to an environment altered by endogenic or exogenic compounds. Changes in the activities of enzymes like glutathione-s-transferase (GST) EC 2.5.1.18, glutathione reductase (GR) EC 1.8.1.7 and glutathione peroxidase (GPx) EC 1.11.1.9 are regarded as the fast and prognostic indices for the environmental stress and used to predict the effects of contaminants on the animals, ecosystems and humans. These enzymes can be induced by ROS and they may be useful indicators of oxidative stress.

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AIMS AND OBJECTIVES

In the present study acute toxic potential of acid blue-113 (AB-113) (CI: 26360), was evaluated in the fry (very sensitive stage) of two Indian major carps, *L. rohita* and *C. mrigala*. These fishes are found abundantly in the rivers of India and are cultivated at a large scale in Punjab. Mortality and levels of three antioxidative/detoxification enzymes (GST, GR and GPx) in flesh were observed as an indicator of the stress of AB-113 after 96h exposure. Till date hardly any work has been done on the effect of azo dyes on antioxidative/detoxification enzymes of fish, therefore, exploration of these enzymes as an indicator of the toxicity of AB-113 holds great significance as both these are important food fish.

MATERIAL AND METHODS

All AR grade chemicals used for the present study were purchased from Sigma-Aldrich and SRL. Azo dye, AB-113 was purchased from the local market in Amritsar, Punjab, India. Fry of *L. rohita* and *C. mrigala* (0.5-1 cm length and 0.07-0.1 g weight) were collected from the ponds of Government Fish Seed Farm, Rajasansi, Amritsar. The fry were subjected to an acclimation period of three weeks in plastic pools of 200 L capacity in the laboratory. Fish were fed on a mixture of rice bran and oil cake (1:1) during acclimation but no feed was given 24h preceeding exposure and during the bioassay. Water was changed every day, tap water after dechlorination was used as diluents. Semi static bioassays were carried out by exposing the fry of both the fish separately to AB-113. Ten fry were exposed to dye in triplicate for 96h and mortality was recorded at 24h interval. The fry which did not move on prodding with a glass rod was considered to be dead. The doses of AB-113 for *L. rohita* were 0, 0.2, 0.4, 0.6, 0.8, 1.0 and 2.0 mg/L and that for *C. mrigala* were 0, 1.5, 2.0, 2.5, 3.0 and 3.5 mg/L. Test water was renewed every day.

**Enzyme activity**

Level of the three enzymes was estimated in the flesh of the fish after 96h exposure. Live fry were collected, flesh was separated from scales and kept in 0.6% saline. Flesh was dried, weighed and homogenized in a respective cold buffer for the enzyme. Ice was kept around the homogenizing tube to avoid heating and enzyme denaturation. The homogenate was centrifuged at 10,000g for 45 min at +4ºC and supernatant was collected for estimation of specific activity of enzymes (µM/min/mg protein) with the help of a systronics dual beam spectrophotometer model-Genesis-10UV.

GST activity was measured with 1-chloro-2,4-dinitrobenzene as substrate. 10% homogenate was prepared in 0.1 M sodium phosphate buffer (pH 7.6) containing 1 mM phenylthiourea (PTU). Assay was performed in a reaction mixture containing 100 µL of ethanolic CDNB solution, 100 µL of 50 mM GSH solution and 25 µL of crude enzyme solution with 0.1 M sodium phosphate buffer (pH 7.6) containing 0.1 mM PTU in a total volume of 1 mL. Enzyme activity was determined by monitoring the increase in absorbance at 340 nm at intervals of 1 min for a total of 5 min.

GR activity was assayed, with some modification, by measuring the oxidation of NADPH at 340 nm. 20% homogenate was prepared in 50 mM potassium phosphate buffer (pH 7.6). The reaction mixture consisted of 1.2 mL of 50 mM potassium phosphate buffer (pH 7.6), 0.2 mL of 3 mM EDTA, 0.2 mL of 0.1 mM NADPH and 0.2 mL of 1 mM GSSG.

GPx activity was determined by employing H₂O₂ as substrate. 20% homogenate was prepared in 0.1 M sodium phosphate buffer (pH 7.0) containing 5.0 mM EDTA. The reaction mixture consisted of freshly prepared GR solution (2.5 U mL⁻¹) in 0.1 M sodium phosphate buffer (pH 7.0) containing 5 mM EDTA, 10 mM sodium azide, 1.6 mM NADPH, 4 mM H₂O₂ and 10 mM GSH. The oxidation of NADPH was followed at 340 nm at intervals of 1 min for a total time of 5 min. Concentration of protein in the extract was measured with Bovine serum albumin as standard.

**Statistical analysis**

Data for mortality were subjected to probit analysis for calculation of 96h LC₅₀, fiducial limits, safe application rate (SAR) and regression equations of AB-113 for the...
Experimental fish. Data were subjected to one-way ANOVA and Tukey test for finding out the differences in the levels of enzyme after exposure and among the activity of enzymes. The biochemical results are reported as Mean±S.E. The differences were regarded as statistically significant when $P<0.001$.

RESULTS AND DISCUSSION

Table 1 shows the values for 96h LC$_{50}$, fiducial limits, SAR, other statistical values and regression equations of AB-113 for L. rohita and C. mrigala. 96h LC$_{50}$ of AB-113 came to be 0.54 mg/L for L. rohita and 1.73 mg/L for C. mrigala. Exposure to the dye intensely affected behavior of the fry as they became restless and tried to jump out of the water, the response was dose-dependent. Gradually the fish stopped swimming and remained static in a corner of the aquarium. Intermittently fish swam unsteadily with jerky movements and turned upside down before mortality. Mucus secretion increased in the exposed fish and dead fish had a thick coat of mucus on the body and gills. Color of the body and gills became blue on exposure to higher doses of AB-113.

<table>
<thead>
<tr>
<th>Fish</th>
<th>96h LC$_{50}$ (mg/L)</th>
<th>Variance (Vm)</th>
<th>S.E.</th>
<th>Fiducial limit upper</th>
<th>Fiducial limit lower</th>
<th>$\chi^2$</th>
<th>SAR (mg/L)</th>
<th>Regression equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. rohita</td>
<td>0.54</td>
<td>0.02</td>
<td>0.13</td>
<td>0.98</td>
<td>0.27</td>
<td>0.09</td>
<td>0.03</td>
<td>y=6.58x+1.78</td>
</tr>
<tr>
<td>C. mrigala</td>
<td>1.73</td>
<td>0.0031</td>
<td>0.0560</td>
<td>2.22</td>
<td>1.34</td>
<td>0.2</td>
<td>0.6</td>
<td>y=4.903x+1.064</td>
</tr>
</tbody>
</table>

Enzyme assay

Data given in Table 2 shows the activities of GST, GR and GP$_x$ in the flesh of L. rohita after 96h exposure to AB-113. There was a significant ($P<0.001$) dose dependent increase over control in the activities of the three enzymes. GST and GR activity showed a continuous increase over control while GP$_X$ activity declined over control in 0.2 mg/L dye but thereafter it increased dose dependently. Table 3 shows changes in the activity of the three enzymes in the flesh of C. mrigala after 96h exposure to AB-113. Exposure to various doses of the dye brought a significant ($P<0.001$) decline in the activity of GST, GR activity showed a continuous increase ($P<0.001$) over control while GP$_X$ activity increased ($P<0.001$) over control in 1.5 mg/L dye but then showed a dose dependent decline. Enzyme activity could not be measured in 2.0 and 3.5 mg/L dye for L. rohita and C. mrigala, respectively as all the exposed fish died within 96h exposure.

Table 2 : Activity of GST, GR and GP$_X$ ($\mu$M/min/mg protein) in the fry of L. rohita after 96h exposure to AB-113

<table>
<thead>
<tr>
<th>Conc. (mg/L)</th>
<th>GST Mean±S.E.</th>
<th>GR Mean±S.E.</th>
<th>GP$_X$ Mean±S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>2.05±0.06$^a$</td>
<td>0.31±0.03$^a$</td>
<td>1.98±0.07$^{ab}$</td>
</tr>
<tr>
<td>0.2</td>
<td>2.51±0.06$^b$</td>
<td>0.45±0.06$^{ab}$</td>
<td>1.47±0.12$^a$</td>
</tr>
<tr>
<td>0.4</td>
<td>2.74±0.07$^b$</td>
<td>0.70±0.03$^{bc}$</td>
<td>2.80±0.17$^{bc}$</td>
</tr>
<tr>
<td>0.6</td>
<td>3.02±0.04$^c$</td>
<td>1.03±0.10$^c$</td>
<td>3.54±0.27$^c$</td>
</tr>
<tr>
<td>0.8</td>
<td>3.85±0.06$^d$</td>
<td>1.54±0.07$^d$</td>
<td>4.37±0.05$^d$</td>
</tr>
<tr>
<td>1.0</td>
<td>3.90±0.04$^d$</td>
<td>1.84±0.13$^d$</td>
<td>5.41±0.24$^e$</td>
</tr>
</tbody>
</table>

Note : Values with different superscript are significantly different at $P<0.001$
The fish, as a biondicator species, plays a very important role in the monitoring of water pollution, because it responds with great sensitivity to changes in the aquatic environment. Fish is extremely sensitive in terms of behavior also and any change in behavior of fishes is related with the toxicity of the chemical. The biomarkers are measurable responses of an organism to the exposure of xenobiotics. Antioxidant enzymes can be induced under conditions of oxidative stress, as an important adaptation to xenobiotic-induced stress.

96h LC\textsubscript{50} of AB-113 for L. rohita was almost 1/3 of the LC\textsubscript{50} value for C. mrigala, which clearly indicates that L. rohita is more sensitive to the stress of this dye as compared to C. mrigala. There is a variation in the toxicity of various dyes to fish as well as there is a variation in the sensitivity of different species of fish to the same dye. 96h LC\textsubscript{50} value of Methyl red for Poecilia reticulata has been observed earlier to be 24 mg/L. On the other hand, 48h LC\textsubscript{50} of C.I. Acid violet 66 and C.I. Acid red 217 was observed, respectively to be 8.20 and 71.04 mg/L for Oncorhynchus mykiss. A dose dependent restlessness followed by no movement at all along with loss of equilibrium observed in the exposed fry can be considered due to the stress of the dye. Behavioral alterations like erratic swimming and restlessness have also been observed in Clarias gariepinus exposed to industrial effluents. After some time the exposed fry of both L. rohita and C. mrigala moved to the corners of the test chambers which can be viewed as an avoidance behavior towards the dye. Fish swim laterally and vertically for some time and then turned upside down before mortality, this may be due to failure of lateroaquostic or neuromast system. Similar observations were recorded when L. rohita was exposed to azo dye Direct green 6. In the present study also a dose dependent blue color on the gills can be correlated with the absorption of the dye leading to a dose dependent increase in mortality of the fish. A thick coat of mucus was noticed on the gills and body of dead fish, coagulation of film anoxia could also be responsible for mortality of fish. Copious mucous secretion was observed in H. fossilis treated with dimethoate.

Dose dependent increase in the activity of GST in the flesh of L. rohita could probably be due to a metabolic adaptation of the fish to the stress of the present dye. An increase in the activity of GST in azo dye exposed fish has also been observed earlier. GSTs catalyze conjugation of both endogenous substances and xenobiotics with glutathione for their excretion from body in less toxic form, therefore rise in their activity clearly indicates that AB-113 is very toxic to L. rohita as it is able to induce this enzyme even at a very low dose i.e. 0.2 mg/L. On the other hand continuous dose dependent decline in the activity of GST in the flesh of C. mrigala may also be directly related to the stress of the dye as the concentrations of the dye for C. mrigala in the present study were much higher than L. rohita.

Dose dependent increase in GR in both the fish can be directly related to the stress of present dye on the defense mechanisms, as increased activity of GR is an indicator of accumulation of GSH under stress as GR generally increases to recycle it. Increased GSH levels as well as
elevated GR activities in liver, kidney and brain of *Carassius auratus* have been observed and GSH metabolism has been suggested as playing a critical role in cell protection against the deleterious effects of chromium. However, fluctuation in GR activity was observed in Tartrazine treated rats, while an increase in GR activity has been observed in NaClO exposed carp and North sea oil exposed Atlantic cod.

Increase over control in GPx activity of *L. rohita* and decline in GPx activity in *C. mrigala* directly indicates that the dye strongly affects the metabolism of the fish and alters the levels of enzymes differently in different species. GPx is an antioxidative enzyme involved in the detoxification of ROS therefore it plays an important role in defense against lipid peroxidation. It seems that the present dye induced ROS generation in the fish which was in turn responsible for an increase in the level of GPx in the flesh of *L. rohita* and its depletion in *C. mrigala* with an increase in dose. GPx is involved in neutralization of higher levels of lipid hydroperoxides produced by superoxide radicals (O$_2^-$) therefore till 1 mg/L dose there is an increase in the level of GPx (*L. rohita*). Fluctuations in GPx activity in the testis of Tartrazine treated rats observed by Visweswaran. Low level of the enzyme in the dye exposed *C. mrigala*, however, hints towards inefficiency of the fish in neutralizing the impact of peroxides at the higher doses of the present dye (1.5 mg/L-3.0 mg/L dye) as observed earlier.

**CONCLUSION**

The results of this research indicate that Acid blue-113 azo dye is very toxic to fish, especially *L. rohita* is very sensitive (96h LC$_{50}$<1 mg/L) to the present dye in comparison to *C. mrigala* (96h LC$_{50}$=1.73 mg/L). Short exposures to even low doses of the dye alter behavior of the fish and absorption of the dye seems to be responsible for dose dependent increase in the mortality of fish. A thick coat of mucous on the body and gills of fish can also be linked to mortality under the stress of the present dye. Activity of antioxidative/detoxification enzymes changed in the flesh of both the fish under the influence of dye but maximum alteration was observed in the activity of GR. Therefore GR can act as an effective indicator for the stress of the present dye.

**ACKNOWLEDGEMENT**

Financial support from University with Potential for Excellence (UPE), University Grants Commission, New Delhi, India is greatly acknowledged.

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